GLOBAL REVIEW OF RECENT TAXONOMIC RESEARCH INTO *ISOETES* (ISOETACEAE), WITH IMPLICATIONS FOR BIOGEOGRAPHY AND CONSERVATION[1]

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ABSTRACT

With the advent of cytological analysis, Scanning Electron Microscopy, the recognition of sterile hybrids, improved access to wild populations and increasingly comprehensive molecular investigations, there has been a revolution in *Isoetes* taxonomy in recent times. The last 40 years have seen an almost 100% increase in the diversity documented during the previous two centuries. Four geographic areas account for over 90% of this diversity: northern-central South America, eastern North America, western Eurasia (including the northern Mediterranean) and South to Tropical Africa. There is a significant global conservation concern for *Isoetes*, with many taxa being rare (known from one to a handful of populations) and some known only from herbarium specimens. The taxa of the extraordinary rock-outcrop pools and the ephemeral wetlands found in disjunct areas around the world appear to be particularly vulnerable to habitat destruction. We expect 100 or more new taxa to be described in coming years. Such taxonomic productivity will require more systematic cytological and molecular investigations to be undertaken. It also requires the assembly of a larger global inventory of high-quality voucher specimens to provide the material for such investigations.

INTRODUCTION

Speaking of quillworts (*Isoetes*, Isoetaceae), the late geneticist and pteridologist, Donald Britton, said only half-jokingly "…. *this is a crazy genus! Just add water and stir*" (D. M. Britton in lit, 1989). In the following, we undertake a review of the dramatic changes that have occurred in our global understanding of the taxonomy of this 'crazy genus' in recent decades as a result of the work of Britton and many others.

Isoetes (Isoetaceae) is an ancient heterosporous lycophyte dating back to the Jurassic Period (Pigg, 2001). With few exceptions, species exhibit a similar simple form and structure (Figure 1). A spirally-arranged, typically small (3 - 30 cm tall), set of

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[1] The authors dedicate this paper to the memory of Donald M. Britton (1923-2012) and Enrico Bellini (1940-2002) who in addition to being fine gentlemen and scholars, inspired and developed our interest in *Isoetes*.

unbranched quill-like leaves with usually partially velum-covered sporangia embedded into their basal abaxial faces, is developed from a shallowly buried two or three-lobed corm. At a gross scale there is not much to separate one taxon from another. Just the same, a high degree of taxonomic diversity as well as a high degree of endemism (Jermy, 1990) exists with approximately 200 species, subspecies and varieties currently recognized (Troia et al., 2016).

Spore morphology is the primary character employed for distinguishing individual *Isoetes* taxa. The megaspores demonstrate a remarkable diversity of perispore (surface) ornamentation expressions (Figure 2), ranging from laevigate (virtually featureless) to tuberculate (covered in broad, low, symmetrical projections) to echinate (spiny) or cristate-reticulate (intricate patterns of muri (walls), tubercles and/ or spines) (Hickey, 1986b; Punt et al., 2007). These ornamentation patterns are typically expressed consistently throughout the range of each taxon.

The notable exception to consistent morphological representation is with sterile hybrids. The megaspores of such taxa have been the source of much confusion for over a century (Engelmann, 1882; Motelay, 1892; Eaton, 1900), exhibiting a range of sizes and morphological features intermediate between their putative parents. Deformed (often flattened) individual megaspores with congested ornamentation patterns likened to brain coral and/ or with irregular, even 'dumb-bell' shapes (Figure 3) are frequent and are strongly indicative of sterile hybrid status.

Microspores are typically less boldly ornamented, varying from plain to tuberculate to echinate (Figure 4). While consistent in size in relation to the cytology of the particular taxon (Kott & Britton, 1983; Taylor et al., 1993; Musselman, 2002; Macluf et al., 2006), they often vary widely in appearance, even within individual populations. Microspores of hybrids are variable in size. Typically they are amphibious and misshaped and, as with megaspores, exhibit ornamentation patterns intermediate between those of the putative parents.

All *Isoetes* occupy sites which are at least seasonally wet and grow in fresh (rarely brackish) water, typically being amphibious or purely aquatic, most growing in sterile or at least nutrient poor, typically acidic to circumneutral substrates. These habitats vary widely from deep-water oligotrophic lakes (growing as deeply as 4-5 metres), permanently flowing rivers and high elevation bog pools, to open woodland seepages, shallow ephemeral swales and briefly inundated pools in open bedrock outcrops.

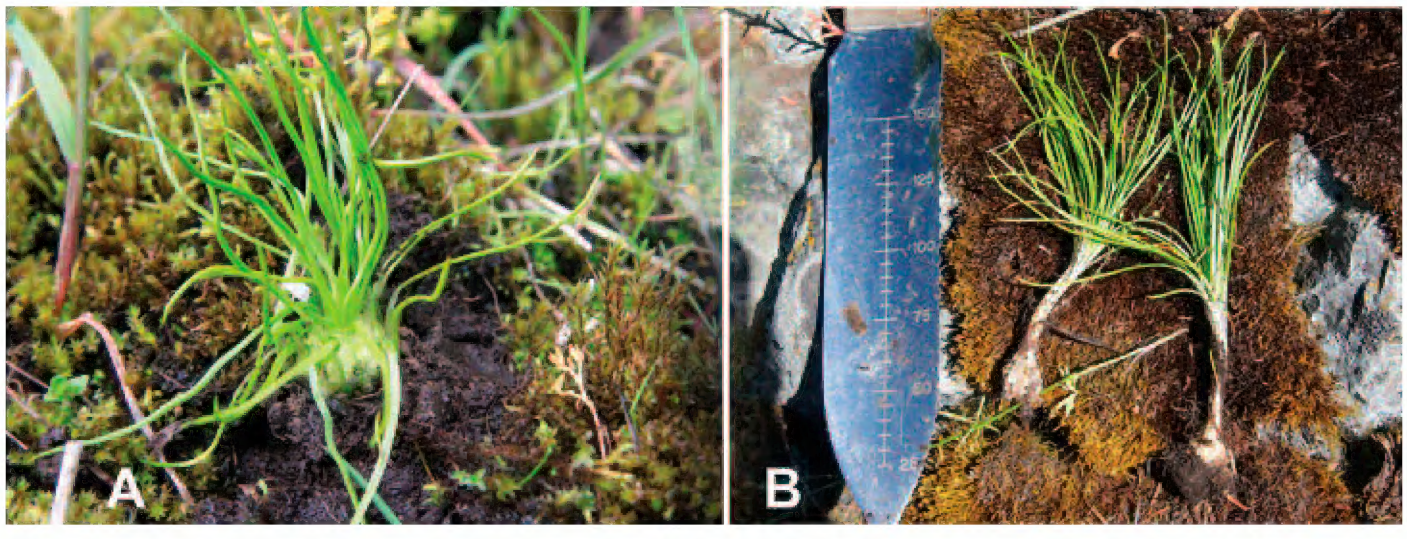

Figure 1: A typical *Isoetes* plant: A) in situ, and B) excavated, also showing corm and roots (*Isoetes minima* A.A.Eaton, Salmo, British Columbia, Canada, 11 June 2017 and 28 June 2008, respectively) (Photos: D. F. Brunton).

Through all of this, be they like the more than half metre long, flexulose aquatic species *Isoetes japonica* A.Braun of eastern Asia, the more typically less than 30 cm long tall, reflected, emergent *I. drummondii* A.Braun of southern Australia, or the stiffly-erect, 5-15 cm tall rock pool ephemeral *I. piedmontana* (N.Pfeiffer) C.Reed of the southeastern United States (USA), virtually all *Isoetes* retain this simple gross-scale

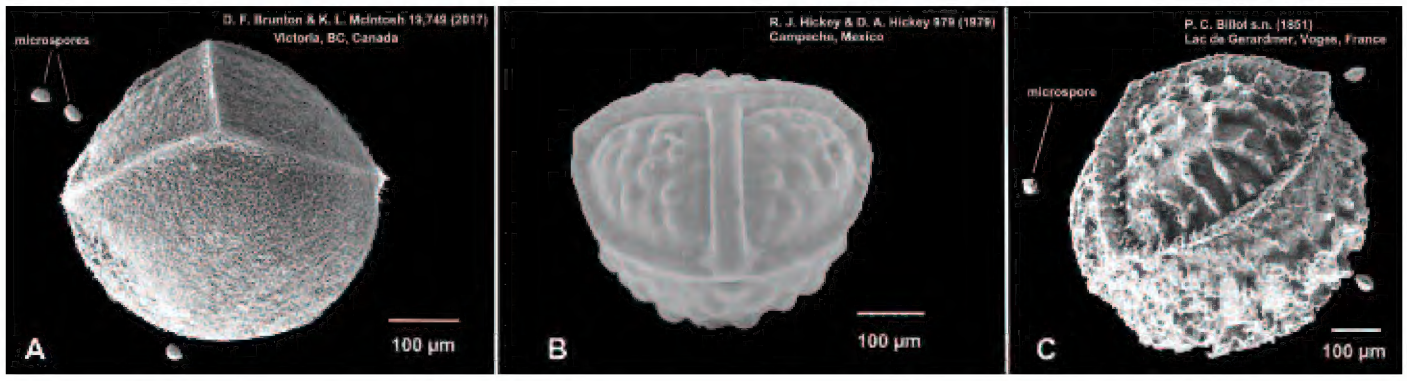

Figure 2: Variations in *Isoetes* megaspore ornamentation: A) diploid *I. nuttallii* A.Braun, B) diploid *I. cubana* Engelm., C) decaploid *I. lacustris* L.(s. str.). (SEM images A & C - P. Sokoloff; B - D. M. Britton).

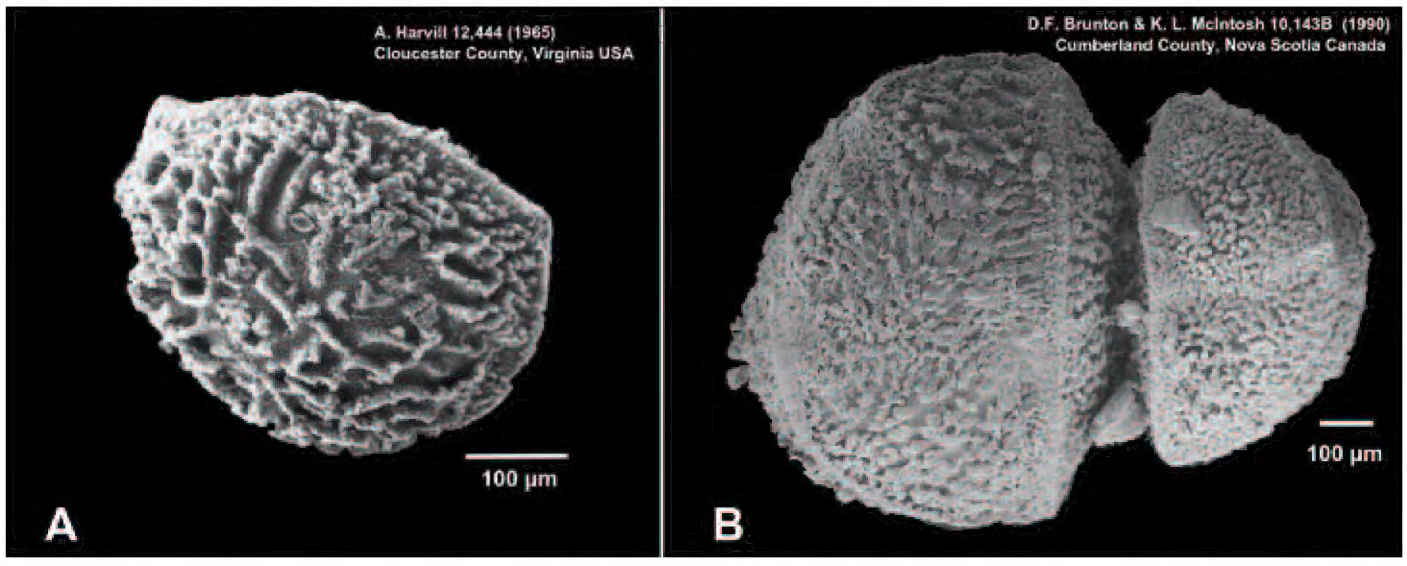

Figure 3: Aborted, sterile *Isoetes* hybrid megaspores: A) triploid *I. engelmannii* A.Braun x *hyemalis* D.F.Brunton, b) heptaploid *I.* ×*harveyi* A.A.Eaton, pro sp. (*I. echinospora* ssp. *muricata* (Durieu) Löve & Löve x *lacustris*) (SEM image A - P. Sokoloff; B - D. M. Britton).

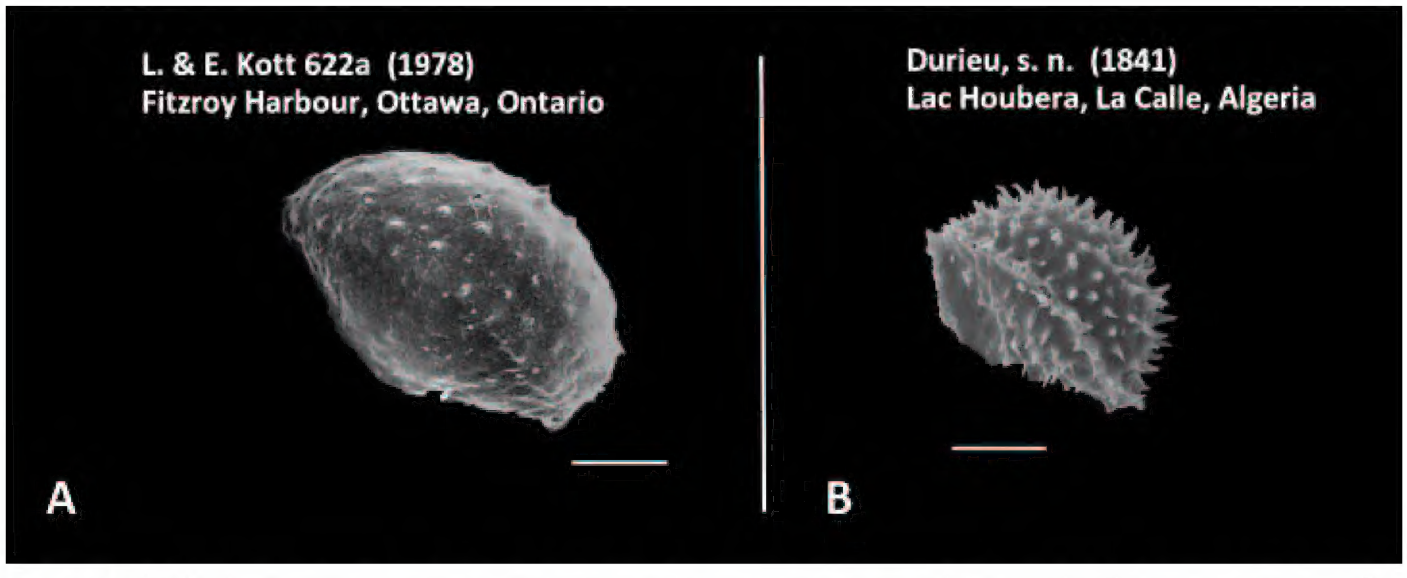

Figure 4: Variation in *Isoetes* microspore ornamentation: A) tetraploid *I. septentrionalis* D.F.Brunton., B) *I. longissima* Bory (s. str.) (SEM image A - P. Sokoloff; B - Carmela Di Liberto, University of Palermo, Italy (with Angelo Troia). (Scale bar = 10 µm)

appearance.

There has been a substantial change in our global understanding of the taxonomic diversity of *Isoetes*, however, since it was last enumerated almost 100 years ago (Pfeiffer, 1922). Change has been particularly dramatic in the latter half of that period (Troia et al., 2016), which is the focus of this review. For our purpose, 'recent' commences ca. 1980 when Scanning Electron Microscope (SEM) and cytology were first applied in comprehensive reviews of *Isoetes* in North America (Boom, 1979; Kott, 1980; Kott & Britton, 1983), Europe (Berthet & Lecoq, 1977) and Australia (Marsden, 1979).

METHODS

The following assessments are based upon a comprehensive reading of the *Isoetes* literature as well as the authors' experience from investigations of the genus since the 1980s. This includes on-site ecological and population investigations in southern Europe (A.T.) and across North America (D.F.B.). In concert with various research associates and supplemented by extensive investigations of herbarium material, SEM imagery and the application of cytological data, these studies have clarified or described a substantial number of taxonomic issues (see *References*, below). We pay special attention to the literature concerning systematics, phylogeny, karyology and biogeography. We also considered the literature in other areas of investigation such as ecology, vegetation science, physiology and conservation biology. For the nomenclatural purposes of the present study we follow Troia et al. (2016).

DISCUSSION

Historical Context

Pfeiffer (1922) conducted the most comprehensive global review of the genus, producing a mostly herbarium-based enumeration of the traditional 19th Century - early 20th Century understanding of *Isoetes* diversity. Her study advanced the more modest reviews of Baker (1880) and Motelay (1883) and relied especially upon the work of Braun (1846), Durieu (1864), Engelmann (1867; 1888) and Eaton (1900). Working independently, she described 77 species and varieties globally. The publication of her remarkably insightful review coincided with an independent description of 15 new South American species (Weber, 1922), resulting in a total of 92 taxa being accepted at a species, subspecies or varietal level by the end of that year. That fewer than 25 species were described worldwide in the subsequent 50 years indicates how the technology and raw data available to researchers in that earlier era had largely been exploited by Pfeiffer's time.

After a long period of relative nomenclatural inactivity following Pfeiffer (1922), *Isoetes* has experienced a surge in taxonomic innovations. In effect, there has been a late 20th Century - early 21st Century *Isoetes* taxonomic 'renaissance'. This results in part from geographically expanded investigations of previously poorly explored regions and habitats. More substantially, however, it reflects the application of new investigative tools for the reconsideration of both long-standing interpretations and new data.

Cytology and SEM

Following the pioneering work of Manton (1950) on various pteridophyte genera, the connection between chromosome number and *Isoetes* spore size was investigated in detail in northern North America (Kott, 1980; Taylor et al., 1985; Taylor & Hickey, 1992). These break-through investigations provided confident determination of both the consistency and taxonomic significance of physical distinctions that had previously been

thought to represent only insignificant morphological variations within broadly circumscribed species. Cytological data quickly became a major line of evidence in subsequent global taxonomic investigations.

Cytological investigations revealed that genetically distinct polyploid taxa are common in *Isoetes,* derived from basic diploid species (2x=2*n*=22) through hybridization and subsequent chromosome doubling (Taylor et al., 1985; Taylor & Hickey, 1992; Taylor et al., 1993). Significant progress was made in documenting *Isoetes* cytology in this period, with chromosome counts for over 80 taxa reported in Takamiya (1999) alone. The chromosome numbers for over 46% of all named taxa are presently known (Troia et al., 2016).

The production of detailed, clear illustrations of seemingly subtle variations of various *Isoetes* morphological features through the systematic application of SEM became common in the 1980s and 1990s (Kott & Britton, 1983; Prada, 1983; Ferrarini et al., 1986; Schelpe & Anthony, 1986; Srivastava et al., 1993; Musselman & Knepper, 1994). For the first time this permitted commonly occurring but minute features to be confidently and reliably related to larger-scale characteristics such as velum size, sporangium shape and colouring, sporangium-topping ligule characteristics and site ecology.

In the late 1980s the application of cytological data and SEM imagery in concert with comprehensive reviews of preserved herbarium material and significantly expanded field investigations, lead to the detection and description of sterile hybrids in North America. The existence of natural *Isoetes* hybrids had been identified in Canada 50 years earlier (Jeffrey, 1937) in a publication that was overlooked by researchers for some decades thereafter (Britton & Brunton, 1992). Independently, Matthews and Murdy (1969) reported probable naturally occurring hybrids in the southeastern USA. Other possible hybrids were subsequently reported in the eastern USA (Boom, 1979, Taylor et al., 1985). The first sterile hybrid *Isoetes* to be formally named was *I.* ×*hickeyi* W.C.Taylor & N.Luebke (Taylor & Luebke, 1988).

The certain identification of sterile hybrids is taxonomically important. Their existence confirms that genetic barriers are in place between previously described and morphologically similar species. They also indicate the existence of previously unsuspected species. Although research has not been undertaken evenly throughout the globe, over a dozen hybrids have now been formally described in North America (Taylor et al., 1985; Taylor & Luebke, 1988; Britton & Brunton, 1989; Musselman & Knepper, 1994; Brunton, 2015), with others named in Japan (Takamiya et al., 1997), Russia (Mochalova et al., 2015) and India (Singh et al., 2018). Undescribed sterile hybrids have been detected in western Europe (A. C. Jermy, pers. comm., Prada & Rolleri, 2003; D.F.B., pers. obs.) and northern South America (J. R. Hickey, pers. comm.) and are suspected in South Africa (Musselman & Roux, 2002), Australia (Tasmania) (pers. obs.) and New Zealand (Hofstra & de Winton, 2016).

A Case Study of Recent Diversity

The combination of cytological data, SEM imagery and hybrid detection has confirmed the existence of distinct taxa previously thought only to represent clusters of populations demonstrating insignificant morphological variation within broadly circumscribed species. The widespread North American *Isoetes engelmannii* s.l provides an excellent example of how dramatic (and complex) this recent clarification has been. The specific distinction of diploid *I. engelmannii* s.str. (Figure 5A and C) and *I. valida* (Engelm.)

W.Clute (Luebke, 1992, Brunton & Britton, 1996a) was confirmed by the recognition of their sterile diploid hybrid *I.* ×*haltonharvillii* Musselman & R.Bray (Musselman et al., 1995). A widespread tetraploid (4x=2*n*=44) derivative, *I. appalachiana* D.F.Brunton & D.M.Britton (Brunton & Britton, 1999) was subsequently described (Figure 5B and D), apparently the fertile product of doubled *I.* ×*haltonharvillii* (Brunton & Britton, 1997).

The publication of *Isoetes appalachiana* was followed in turn by the determination of hexaploid (6x=2*n*=66) *I. microvela* D.F.Brunton, suspected to be the fertile doubling of triploid (3x=2*n*=33) *I. appalachiana* x *engelmannii* (Brunton & Britton, 1998). Tetraploids *I. hyemalis*, *I. riparia* Engelm. s.str. and *I. septentrionalis* (Brunton et al., 1994, Caplen & Werth, 2002; Brunton & McNeill, 2015), as well as hexaploids *I. boomii* N.Luebke and *I. georgiana* N.Luebke (Luebke, 1992; Brunton & Britton, 1996b), all represent recently described or clarified taxa within or connected to *I. engelmannii* s.l.

Three additional sterile hybrids were identified in this period from within the *Isoetes engelmannii* s.l. species complex: tetraploids *I.* ×*brittonii* D.F.Brunton & W.C.Taylor (*I. appalachiana* x *septentrionalis*) (Brunton & Taylor, 1990; Brunton, 2015), *I.* ×*bruntonii* D.Knepper & Musselman (*I. appalachiana* x *hyemalis*) (Musselman et al., 1996; Brunton, 2015) and hexaploid *I.* ×*fairbrothersii* J.D.Montgomery & W.C.Taylor (*I. engelmannii* x *lacustris*) (Montgomery & Taylor, 1994). In addition, 19th Century taxa *I.* ×*foveolata* A.A.Eaton, pro. sp. (*I. engelmanii* Dur. x *tuckermanii* A.Braun) and *I.* × *eatonii* R.Dodge, pro. sp. (*I. echinospora* x *engelmannii* and its synonym *I.* ×*gravesii* A.A.Eaton, pro sp.) were recognized to represent sterile *I. engelmannii* hybrids (Taylor et al., 1985; Jermy, 1990). Thus since the mid 1980s, over a dozen taxa in eastern North America were recognized to have been derived in whole or in part from *I. engelmannii* s.l.!

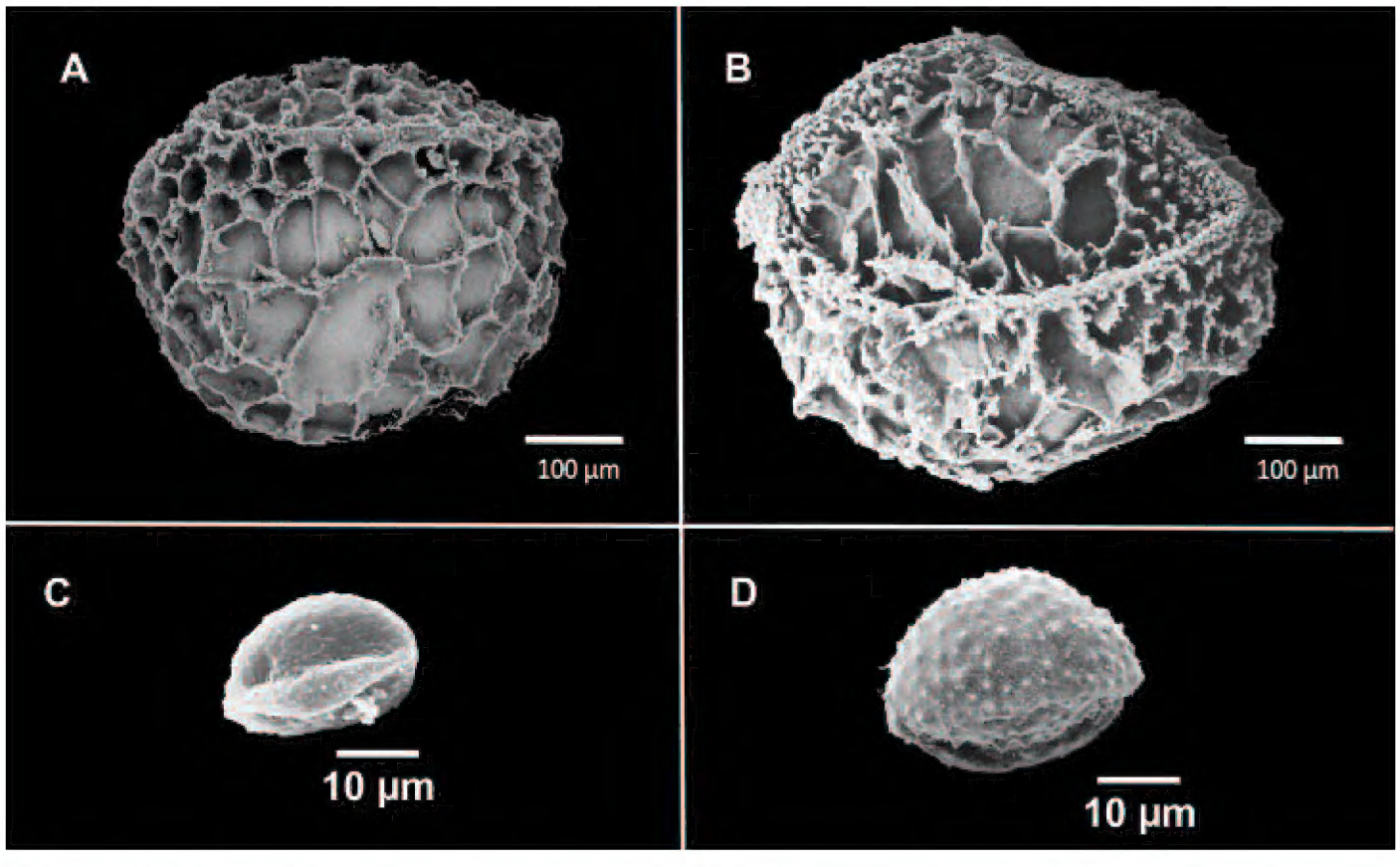

Figure 5: Megaspore (A) and microspore (C) of diploid *Isoetes engelmannii* (St Louis, Missouri, USA, G. Engelmann s. n., 1842 (MO 100901 - Syntype) compared with megaspore (B) and microspore (D) of tetraploid *I. appalachiana* (Huntington County, Pennsylvania, D. F. Brunton & K. L. McIntosh 11,171, 6 July 1992) (SEM images - D. M. Britton).

Recent Diversity Trends

Approximately 80 new taxa have been described in the recent era (Troia et al., 2016). The early to mid 1980s was a particularly productive time with over 20 species or subspecies being described globally, followed by eight new taxa in 1986 and four in each of 1991 and 1994 (Figure 6). A second even larger surge in taxonomic activity occurred early in the 21st Century (almost 25 taxa), with five new taxa recognised in each of 2005 and 2010.

Figure 7 illustrates our contemporary understanding of *Isoetes* diversity, with four global 'hot spots' centred on 1. northern-central South America (64 taxa), 2. eastern North America (45 taxa), 3. western Eurasia (including the northern Mediterranean) (39 taxa), and 4. South to Tropical Africa (35 taxa) (adapted from Troia et al., 2016). Together these account for over 180 taxa, or more than 90% of global diversity. That is a radically different picture from the traditional global understanding of *Isoetes* diversity. Based on

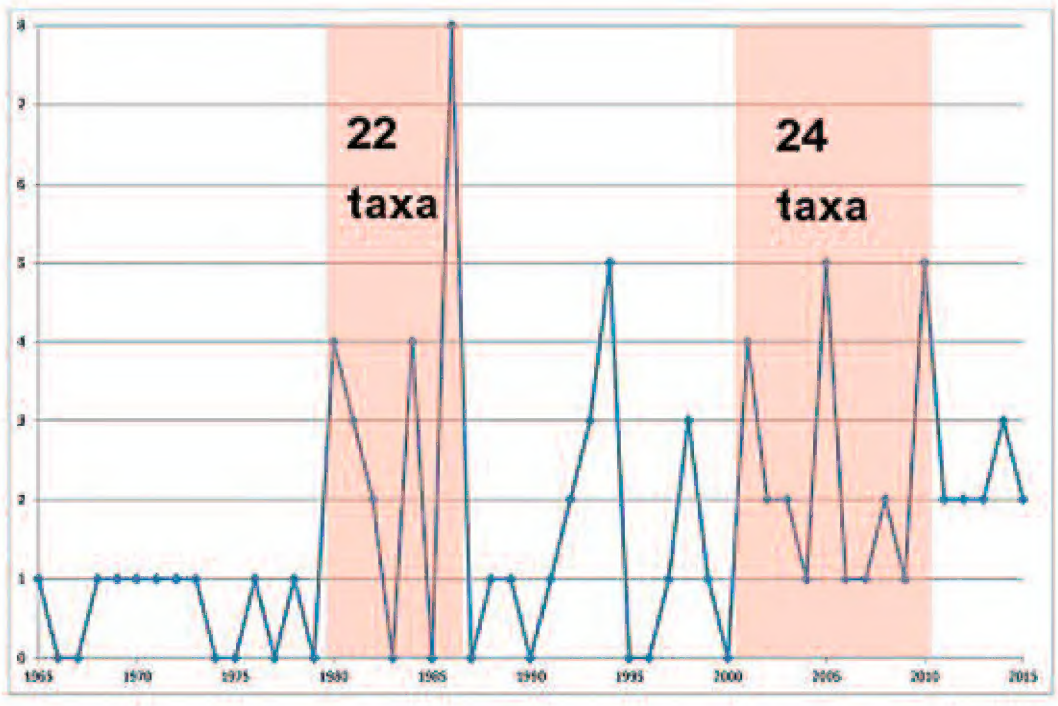

Figure 6: taxonomic activity (number of taxa described) since 1960s (adapted from Troia et al. 2016); particularly active periods shaded.

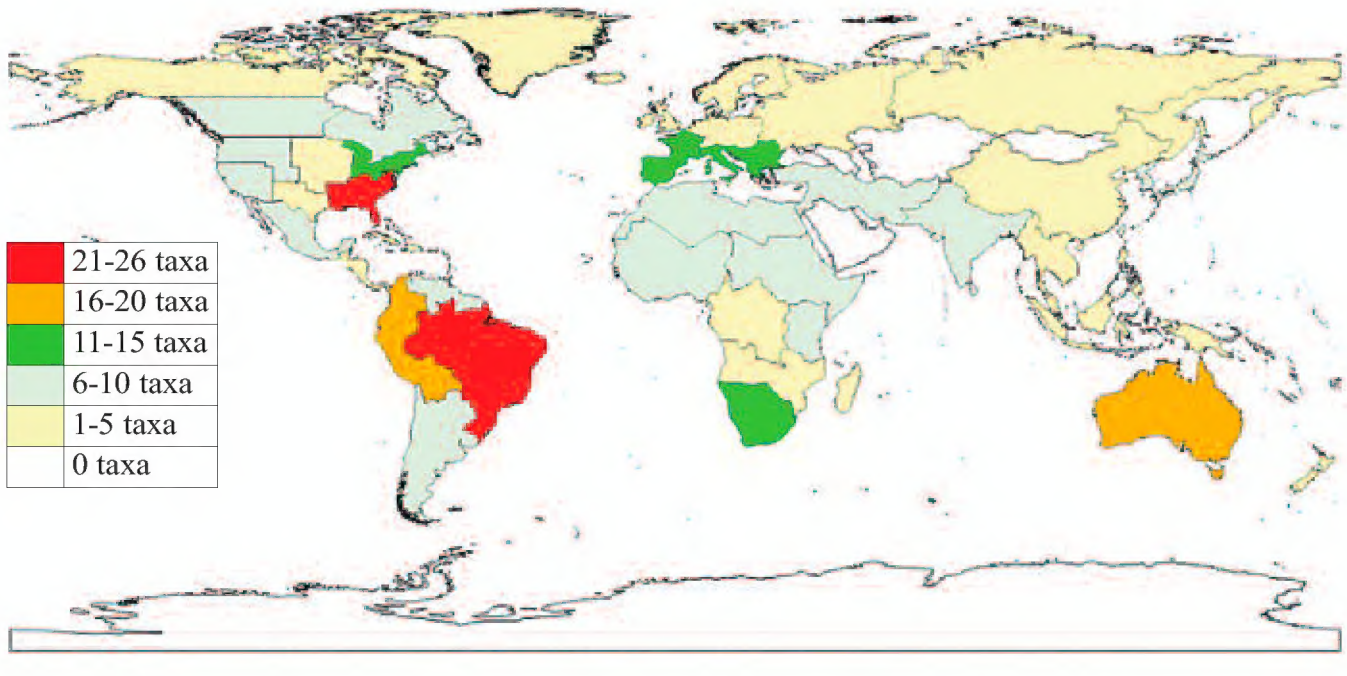

Figure 7: Global *Isoetes* distribution centres of diversity (Troia et al., 2016).

Pfeiffer (1922) and Weber (1922), the traditional five primary centres of diversity would have been identified as western Eurasia (including the northern Mediterranean) (19 taxa - 21% of global diversity), western North America (16 taxa - 17% of global diversity), eastern North America (12 taxa - 13% of global diversity), northern South America (12 taxa - 13% of global diversity), and Australia-New Zealand (9 taxa - 10% of global diversity).

While the prominence of these contemporary 'hot spots' is undoubtedly biased towards regions where 'Isoetologists' have lived and/ or could most easily access, we believe the overall pattern at least broadly represents global reality. Recent developments within the major *Isoetes* diversity areas identified in Figure 7 are summarized below.

Northern-central South America (31% of global diversity)

The expansion of known documented *Isoetes* diversity in northern South America in recent years is remarkable, with more than a doubling of the number of taxa that were known to exist prior to 1930. Most of these are from Brazil, with a particular focus in the Atlantic Rainforest region where almost 40% of pteridophytes are considered endemic (Prado, Sylvestre & Labiak et al., 2015; Prado, Sylvestre & Pereira, 2015). This total includes at least seven *Isoetes* taxa (Pereira, Mittelbach & Labiak, 2015).

A number of new species represent clarifications of taxa first described (though frequently not legitimately so) in the works of Fuchs-Ekert (1982; 1992). Most new taxa are rare, with several being known from only a single (or handful of) site(s) (Pereira et al., 2016). At least one is known only from 19th Century herbarium specimens (Pereira & Stutzel et al., 2017).

This recent neotropical work was substantially built upon the foundation of taxonomic research established by James Hickey and his associates. Their work focused particularly on species diversity in the northwest of the continent (Hickey, 1984; 1985; 1986a; 1989). The taxonomy of *Isoetes* in the northwestern portion of the continent remains substantially unresolved (Pinto-Zarata et al., 2017), being hampered by the limited availability of herbarium material and a confused nomenclatural history. A number of new species have been described in this region (e.g. five of nine species reported from Peru (Hickey, 1994) have been described since 1979 as well as others in the high elevation *I. karstenii* A.Braun complex (Small & Hickey, 2001)). Undescribed species and hybrids remain to be discriminated and documented, however (R. J. Hickey, pers. comm.).

Eastern North America (22% of global diversity)

The recent expansion of our knowledge of *Isoetes* diversity on the eastern side of the North American continent commencing with the investigation of species diversity in the southeastern USA by Boom (1979; 1982) has been simply explosive. The systematic review of herbarium collections in the 1980s and 1990s and subsequent field investigations supported by cytological analysis of morphologically, ecologically and/ or distributionally distinctive elements detected within long-established species, has led to the recognition of numerous new taxa. While 32 taxa (24 species or subspecies and eight named hybrids) were enumerated in the definitive *Flora of North America* (Taylor et al., 1993), the total now stands at 51 taxa (35 species and subspecies and 16 described hybrids). A number of additional candidate species, subspecies and hybrids are under active investigation. This growth represents an almost 60% increase in total North American *Isoetes* diversity over the last 25 years.

Somewhat akin to the geographic concentration witnessed in the Atlantic Coastal Rainforest region of South America (see *Northern-central South America*, above), the Atlantic Coastal Plain of the southeastern USA is particularly species rich and supports a number of rare and endemic taxa (Reed, 1965; Musselman et al., 2001; Brunton & Britton, 2006; Brunton, 2016).

Considerable attention has been focussed in recent years on species of shallow, ephemeral pools occurring on open, acidic, granitic or sandstone bedrock outcrops (flatrocks) in the southeastern USA (Matthews & Murdy, 1969; Heafner & Bray, 2005; Brunton, 2016). Comparable species have been described from central and southern Africa (Hall, 1971; Schlepe & Anthony, 1986; Roux et al., 2009), the Mediterranean region (Troia & Raimondo, 2009; Troia & Greuter, 2015b), southern and central Australia (Johnson, 1984; Chinnock, 1993; 1998) and Mexico (Mora-Oivo, 2013) from similar outcrop pools and ephemeral swales. Such pools are otherwise referred to as erosion pits, !gua, gnammas and vleis. These outcrop taxa include the only known distichous-leaved *Isoetes*, the diminutive and remarkably similar *Isoetes tegetiformans* Rury of Georgia, USA (Rury, 1985; Allison, 1993) and *I. australis* Williams of western Australia (Williams, 1943).

Outcrop taxa are frequently rare and are confined to widely disjunct sites. Many species, such as the aforementioned *Isoetes tegetiformans* and *I. australis* or *I. melanospora* Engelm. of the southeastern USA and *I. pusilla* C.R.Marsden & R.J.Chinnock of eastern Australia (Entwhisle, 1994), bear remarkable ecological and morphological similarities (Figure 8) despite being hugely separated geographically.

Western Eurasia (including northern Mediterranean) (19% of global diversity)

Pfeiffer (1922) initially enumerated 13 taxa in this region. Although Berthet & Lecocq (1977) made an important contribution to European *Isoetes* research with their SEM-based review of French taxa, taxonomic reconsiderations in the period began with the work of Carmen Prada (Prada, 1979; 1980; 1983) focussed on the Iberian peninsula. Beyond that, only occasional taxonomic investigations (including karyology) were undertaken (Schneller, 1982; Britton & Brunton, 1996). With the start of the new Millennium, however, dedicated *Isoetes* research was initiated in Italy (Troia & Bellini, 2001; Cesca & Peruzzi, 2001; Peruzzi et al., 2003), including the review and reconsideration of known diversity and the description of new species (Troia & Raimondo, 2009; Troia & Azzella, 2013).

In this period Prada and associates expanded their studies beyond Spain (Prada & Rolleri, 2005; Rolleri & Prada, 2007). Romero and associates focused on critical Spanish taxa (Romero et al., 2004; Romero & Real, 2005) while Musselman and associates (Musselman, 2002; Bolin et al., 2008; 2011) studied *Isoetes* in the eastern part of the Mediterranean basin. Troia and associates concentrated their studies on expanding and clarifying previously documented *Isoetes* taxonomy in the central and eastern Mediterranean region. This included national syntheses for Italy (Troia & Greuter, 2014, 2015a) and Greece (Troia & Greuter, 2015b). In addition, a nomenclatural clarification of the taxonomically important but perplexing *I. longissima-velata* complex of the Mediterranean has been prepared (Troia & Rouhan, 2018).

The treatment of *Isoetes* in *Flora Europaea* (Jermy & Akeroyd, 1993) lists 14 taxa. Jermy also studied aquatic taxa in northwestern Europe, providing the first confirmation of a European sterile hybrid (*I. echinospora* s.str. x *lacustris* - A. C. Jermy, pers. comm.). Documentation was also provided at that time for triploid plants of *I. echinospora* (*s.*

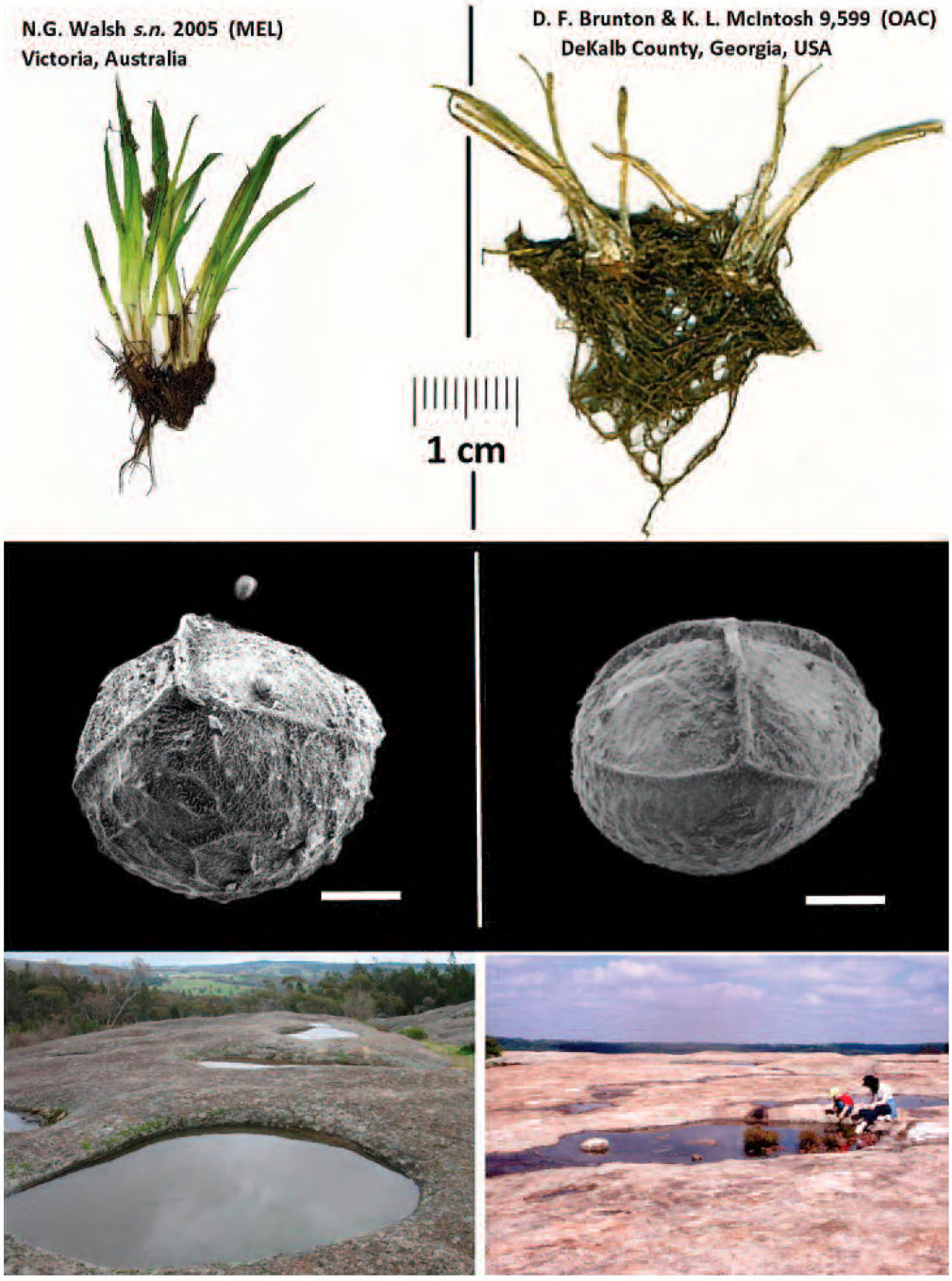

Figure 8: Morphologically similar bedrock outcrop pool species *Isoetes pusilla* of eastern Australia, LEFT (top to bottom): full plant; megaspore and microspore from this specimen (SEM image P. Sokoloff); habitat at Ingram's Rock, Victoria (Photo: N. Walsh), and *I melanospora* of the southeastern USA, RIGHT (top to bottom): full plant; megaspore from this specimen (SEM image D. M. Britton); habitat at Mount Arabia, DeKalb County, Georgia (Photo: D. F. Brunton). Scale bar = 100 μm.

str.) in England that were morphologically indistinguishable from the otherwise diploid plants of this species but showed no evidence of hybridization (Rumsey et al., 1993); this unique circumstance for at least a Northern Hemisphere *Isoetes* species remains unresolved (F.J Rumsey, pers. comm.).

Vegetation inventories of subalpine *Isoetes* communities in the Pyrenees Mountains of France, Spain and Andorra have led to the unexpected discovery and description of *I. creussensis* J.J.Lazare & S.Riba (Lazare & Riba, 2010; Aymerich & Saez, 2013), probably the duodecaploid (12x=2n=132) detected earlier in this region by Taylor & Hickey (1992). A hexaploid (6x=2n=66) or heptaploid (7x=2n=77) *I.* ×*hickeyi*-like sterile hybrid has also has been reported (Taylor & Hickey, 1992) here. A reconsideration of the rare and ambiguous (possibly hybrid) taxon *I. brochonii* M.L.Motelay, and its relationship to other Pyrenean taxa, is on-going (D.F.B., pers. obs.).

South to Tropical Africa (17% of global diversity)

Pfeiffer (1922) enumerated only four taxa in this region, probably because she was limited by the availability of voucher material and the restricted nature of botanical explorations in the region up to that time. That number of documented taxa has increased steadily over the last century, with at least 10 taxa now known. The increase to date is entirely from southern Africa (Wanntorp, 1970; Schelpe & Anthony, 1980; Musselman & Roux, 2002). Most new taxa are found in briefly-flooded ephemeral ponds (vleis) or bedrock outcrop solution pits (Roux et al., 2009).

Tropical African species remain less well known, presumably due to continuing limited field exploration opportunities and relatively limited herbarium collections. A variety of amphibious species in ephemeral grassy wetlands and perhaps also in flat rock-like habitats over sandstone bedrock, are known here (Halls, 1971).

Areas of Lesser Diversity

A number of areas of lesser diversity have also received particular attention in recent years. These are described below in descending order of the documented diversity that is apparent.

Australia - New Zealand (16 - 23 species; 8 to 11% of global diversity): *Isoetes* of that region, along with those in a major area of eastern Asia, were enumerated in a comprehensive investigation by Marsden (1979). In that foundational study a number of new taxa from Australia were described, several of which are confined to ephemeral open bedrock pools (Chinnock, 1993). These share remarkably close morphological and ecological affinities with flat rock species in the southeastern USA (see *Eastern North America*, above). Approximately double the traditional number of Australian *Isoetes* known to Pfeiffer (1922) are now recognized (Chinnock, 1998) - a total of 16 species, with at least one Tasmanian species remaining undescribed (Garrett, 1996). Much remains to be resolved here, however, with possible hybrids and cryptic species suggested by abnormal morphological characteristics in several species (referred to by Marsden (1979) as Type I, Type II and Type III megaspores) and multiple ploidy levels reported from other taxa such as apparently apomictic *I. muelleri* A.Braun s.l. (Chinnock, 1998).

The taxonomy of New Zealand taxa was unresolved in Marsden's (1979) study and remains so today. There appear to be up to five aquatic taxa represented here, probably including hybrids (Hofstra & de Winton, 2016).

Eastern Asia **(Japan/ China/ Korea)** (12 taxa; 6% of global diversity): Research over the last three decades has doubled the known diversity of *Isoetes* taxa in this region.

All the newly described taxa are aquatics and most are very rare (Liu et al., 2005), being known from one to a handful of sites. Two (including *I.* ×*michinokuana* M.Takamiya, M.Watanabe & K.Ono, the first Asian hybrid to be described) result from the especially comprehensive morphological and cytological investigations of Japanese *Isoetes* by M. Takamiya and associates (Takamiya et al., 1997; Takamiya, 2001). Another two new species are found on a single island off the coast of Korea (Choi et al., 2008).

Central America - Caribbean (10 taxa; 5% of global diversity): Known diversity in this floristically transitional area between North and South America is moderate in extent but demonstrates wide ecological variation and significant endemism (e.g. four species confined exclusively to Mexico). Known diversity has increased in recent years with the description of three new endemic species, including *I. jamaicensis* J.R.Hickey from its namesake island (Hickey, 1981), the lowland *I. pallida* J.R.Hickey of the South American *I. triangula* Weber s.l. complex found in southern Mexico (Hickey, 1988), and a bedrock outcrop pool taxon *I. tamaulipana* A.Mora-Olivio, A.Menoza-Ruiz & J.G.Martinez-Ávalos, known from a single site in north-eastern Mexico (Mora-Olivio et al., 2013).

India (6-23 taxa; 3 to 11% of global diversity): Ironically, while the endemic *Isoetes coromandalina* L.f. s.str. was one of the first *Isoetes* to be described (Linnaeus 1781), it was the only known member of the genus from India known to Pfeiffer (1922). The 18 species now reported from the subcontinent (Shukla et al., 2002, Pantil & Rajput, 2017) were almost all described after 1960 (Troia et al., 2016).

The disposition of a number of recently described taxa and the various subspecies and varieties within several species complexes (Pant & Shrivastava, 1962; Gena & Bhardwaja, 1984; Shrivastava et al., 1993; Srivastava, 1998; Shukla et al., 2002) is controversial and remains unresolved. This is due to uncertainties from the reports of multiple and irregular chromosome counts and possibly apomictic reproduction amongst widely scattered, rarely occurring taxa with distinctions dependent on variable morphological characteristics. At least six of the 23 described taxa (Pantil & Rajput, 2017), however, seem solidly established at a species level (Fraser-Jenkins et al., 2016; Troia et al., 2016). The first Indian sterile hybrid, *I. xgopalkrishnae* S.K.Singh, P.K.Shukla & N.K.Dubey, has also recently been described (Singh et al. 2018).

North Pacific Beringia (ca. 7 taxa; 3% of global diversity) - The *Isoetes* taxa of the area between Far East Asia (Siberia, Russia) and northwest North America (Alaska, USA and Yukon, Canada) have received attention in recent years due to their location within the phytogeographically significant Beringian region (Brunton et al., 2015), and one sterile hybrid, *I.* ×*gopalkrishnae* S.K.Singh, P.K.Shukla & N.K.Dubey (Singh et al., 2018) has recently been described. Britton and Brunton (1999), Mochalova (2006) and Mochalova et al. (2015) document at least seven taxa (three hybrids) intermixing across this North Pacific transition zone. One of these, *Isoetes* ×*paratunica* D.F.Brunton, O.Mochalova & A.A.Bobrov (*I. asiatica* (Makino) Makino x *I. maritima* Underw.) (Mochalova et al., 2015), represents the first hybrid reported between putative parents, that occur predominantly on separate continents (Asia and North America, respectively). Research continues on the intercontinental relationships of these taxa and their phytogeographic implications (A. Bobrov, pers. comm.). Kim et al. (2009) and Kim and Choi (2016) have conducted DNA analyses that further refine the origins and taxonomic distinctions of this Beringian complex.

Molecular Investigations

Molecular investigations of *Isoetes* commenced with the Hoot & Taylor (2001) assessment of the value of these data for species determination and the clarification of *Isoetes* phylogeny. Although employed in support of some morphologically and cytologically determined specific taxonomic decisions (e.g. Bolin et al., 2011) and to examine the genetic diversity of particular taxa (Chen et al., 2005), molecular research to date has largely been applied at a higher level. Molecular data have also been employed in many areas of the world over the last 15 years to investigate genetic diversity within and between populations of rare and endangered *Isoetes*. This research has particular importance for the development of conservation management strategies aimed at establishing sustainable populations with such species as *I. hypsophila* H.vonHandel-Mazzetti (Chen et al., 2005; Li et al., 2012), *I. sinensis* T.C.Parker (Kang et al., 2005; Li et al., 2012) and *I. yunguiensis* Q.F.Wang & W.C.Taylor (Chen et al., 2007; Dong et al., 2018) in China, *I. coreana* Y.H.Chung & H.K.Choi (Kim et al., 2008) in Korea and *I. malinverniana* V.deCesati & G.DeNotaris (Gentili et al., 2010; Abeli et al., 2017) in Italy. It has been similarly applied for clarifying diversity in undetermined rare taxa in New Zealand (Hofstra & de Winton, 2016) and with both *I. butleri* G.Engelmann in the United States (Vander Stelt et al., 2017) and *I. durieui* J.Bory in Italy (Troia, 2003). Carrying on from fossil and morphological interpretation-based studies such as Retallack (1997) and Taylor and Hickey (1992), molecular research has focussed on broader questions of phylogeny and the evidence for extinct lineages (Rydin & Wikstrom, 2002; Hoot et al., 2004; Taylor et al., 2004; Larsen & Rydin, 2016; Pereira et al., 2017). This permitted Hoot et al. (2006), for example, to identify six major well-supported *Isoetes* clades globally. In recent years Xiang Lui and associates have applied a significant focus of *Isoetes* molecular research in China on regional origins and prehistoric dispersal patterns (X. Liu, pers. comm.).

Bolin at al. (2018) employed DNA flow cytometry to consider the utility of C Values for the identification of particular species and Freund et al. (2018) combined molecular phylogeny data with morphological documentation to clarify the evolution of particular character states (e.g. the number of corm lobes).

Molecular investigations, both at a high level and in regards to particular taxa represent a new and powerful tool that will be fundamental to future taxonomic studies of *Isoetes*, as they now are to virtually all groups of vascular plants.

Other Areas of Investigation

While the limited scope of our review directs focus onto recent taxonomic accomplishments, it is important to note that significant strides in our understanding of other aspects of *Isoetes* were made during this period. The following briefly notes some of the major achievements of other areas of biological investigation.

Vegetation classification involving *Isoetes* communities was conducted extensively in Eurasia, with considerable work being undertaken recently in the Mediterranean region. A preliminary synthesis of the *Isoetes* - related vegetation classification in this region was made by Quézel (1998). Studies on the ecology and vegetation science of *Isoetes* communities are now available for most (though not all) species around the Mediterranean: northwest Africa (Boutin et al., 1982), Azores (Pietsch, 1994), Spain (Romero & Amigo, 1995; Molina, 2005; Molina et al., 2011), France (Médail, 2003; M. Rhazi et al., 2004; L. Rhazi et al., 2009), Italy (Abeli et al., 2014), Sardinia (Bagella & Caria, 2013) and Turkey (Kürschner & Parolly, 1999). These studies are clearly

conservation-oriented in some cases (e.g. Médail, 2003; M. Rhazi et al., 2004; L. Rhazi et al., 2009; Gentili et al., 2010).

Troia (2016) investigated the dispersal and possible migration routes of some of the current Mediterranean taxa, especially those with African affinities and particularly considering faunal and climatic vectors. Amongst climatic issues, severe wind storms were speculated to be a distributional vehicle for bedrock outcrop *Isoetes* taxa in the southern USA (Brunton, 2001) as they are for other types of terrestrial and wetland vascular plants (Matthews et al., 1991; Brunton et al., 2018).

Presumably discouraged by long-standing taxonomic uncertainty in regards to many species, other contemporary investigations of *Isoetes* biogeography have been relatively few. Studies in East Asia employing geological, cytological and molecular data, however, have examined the origins and original dispersal patterns of the genus in that region (Liu et al., 2004; Taylor et al., 2004). Other investigations on a more local scale considered possible recent Trans-Pacific dispersal between North America and Asia (Brunton et al., 2015).

Ecological and physiological investigations of *Isoetes* have been relatively few, despite being initiated early in the recent era in the pioneering studies of Matthews & Murdy (1969) regarding rock outcrop communities in the southern USA. Indeed, the benefit of considering ecological characteristics as an aid to taxonomic clarification was demonstrated earlier with recognition of the importance of substrate chemistry for distinguishing the morphologically similar ephemerals *I. melanopoda* Gay & Durieu s.str. and *I. butleri* Engelm. in the southern USA (Taylor et al., 1975). Substrate considerations have subsequently been applied to investigations of many aquatic and amphibious species in North America and elsewhere.

Karrfalt and Hunter (1980) examined how the uniquely mucilage-covered sporophylls of South American *Isoetes andicola* (Amstutz) L.D.Gomez (*Stylites andicola* Amstutz) were literally squeezed out of the alpine vegetation in which they developed by the expansion of new plant growth, thus aiding the dispersal of sporangia. Other physiological and morphometric investigations in this period relate to leaf structure (ligule) functions (Kristen et al., 1982; Sharma & Singh, 1984; Singh, 1984), the early development of sporophytes (Hilger et al., 2002) leaf (intrastelar canal) anatomy (Romeo et al., 2000), and the early development of sporophytes (Hilger et al., 2002).

The only extensive *in situ* ecological investigations of purely aquatic *Isoetes* to date were undertaken in central and northern Europe. Margrit Voge conducted underwater studies of numerous deep-water populations of *I. lacustris* at this time (Voge, 1997; 2003). She focussed especially on the importance of water quality parameters for controlling the health and size of particular populations. The importance of water quality to population sustainability was also investigated in China by Wen et al. (2003). More recently, Chappuis et al. (2015) examined the relative importance of light and organic elements in the stability (or instability) of southern European *I. lacustris* populations. Sharma (1998) and Sudova et al. (2011) examined the apparently symbiotic role of mycorrhizal fungi connected with the roots of *Isoetes* taxa in India and central Europe, respectively.

The most dramatic physiological *Isoetes* investigations in recent decades resulted from the remarkable work of Jon Keeley and associates who determined that the CAM photosynthetic process typically employed by desert plants was also widely employed by *Isoetes* (Keeley, 2014). Their field and laboratory studies determined that CO_2 uptake of *Isoetes* plants was at least substantially through their roots, enabling these plants to

prosper in sterile, otherwise prohibitively challenging habitats (Keeley & Busch, 1984; Keeley & Sandquist, 1991). Those investigations focussed mostly on ephemeral pool taxa in semi-arid habitats in southern California, USA but also included subalpine American taxa and South American tropical alpine species (Keeley et al., 1994).

Conservation Biology and Management

Conservation concerns are increasingly significant motivators of *Isoetes* investigations, with many global conservation initiatives focussing on rare species of precarious status that are in danger of extinction. Taxa known from only a single population are known around the world, including *I. pallida* in southern Mexico (Hickey, 1988), *I. viridimontana* M.A.Rosenthal & W.C.Taylor in the northeastern USA (Rosenthal et al., 2014), *I. sabatina* A.Troia & M.M.Azzella (Troia & Azzella, 2013) in central Italy, *I. hallasanensis* H-K.Choi, Ch.Kim & J.Jung in Korea (Choi et al., 2008), *I. libanotica* Musselman, J.F.Bolin & R.D.Bray in Lebanon (Bolin et al., 2011) and *I. junciformis* D.F.Brunton & D.M.Britton in the southeastern USA (Brunton & Britton, 1999). Liu et al. (2005) note that all *Isoetes* taxa in China are in danger of extinction and several eastern Mediterranean taxa are similarly at risk (Bolin et al., 2011; Troia & Greuter, 2015a). Amphibious bedrock outcrop and ephemeral pool taxa may be the most threatened class of *Isoetes* globally, these being considered to be of even greater conservation value by sharing habitat with other specialized (often endemic) flora and fauna (Allison, 1993; Bauder & McMillian, 1998; Bagella & Caria, 2013; Ernandes & Marchiori, 2013; Bagella et al., 2016; Brunton, 2016).

FUTURE POSSIBILITIES

Based on the rate of nomenclatural innovation in recent years (Figure 6) and the incentive provided by new research techniques supplementing traditional databases, we speculate that 100 or more additional *Isoetes* taxa will be identified in future years. The richest, perhaps globally most important areas for investigation would appear to be the bedrock outcrop and ephemeral pool habitats of the USA, Australia and South Africa and the less well investigated areas of Tropical Africa and interior South America. In addition to potentially providing taxonomic insights, the remarkably strong ecological and morphological similarities of geographically widely dispersed bedrock outcrop taxa (Figure 8) suggest an exceptional opportunity for the study of convergent evolution.

Despite no *Isoetes* being known from the area, a strong case can be made for including the Caucasus region of central Asia amongst those having a high potential for future taxonomic contributions. The extraordinarily rich biodiversity of this region is known to support the highest level of Temperate Zone endemism in the Northern Hemisphere (Batsatsachvii et al., 2013).

Molecular clarification of the subtle and even cryptic relationships within *Isoetes* species complexes could have major conservation management implications in many parts of the world. Again, this is especially true in regards to bedrock outcrop and ephemeral wetland taxa.

Particular regions such as India, central and northern South America, South to Tropical Africa, central Asia and the eastern Mediterranean appear to be particularly in need of the initiation or continuation of systematic cytological and molecular taxonomic investigations and clarification. That applies as well to a number of unusually challenging species complexes including *Isoetes muelleri* s.l. in Australia, *I. coromandelina* s.l. in India, *I. longissima* s.l. in the Mediterranean region of Europe, *I. karstenii* s.l. in South

America, and both *I. riparia* s.l. and *I. melanopoda* s.l. in North America.

Although considerable improvements have been seen in recent decades in the growth of *Isoetes* herbarium collections globally, more systematic gathering of high quality material is needed to facilitate future physiological and molecular investigations, particularly with morphologically challenging species groups. This is especially important in geographic regions (identified above) which traditionally have been deficient in preserved plant material. Similarly, cytological determinations remain outstanding for over half of the described taxa (Troia et al., 2017). These determinations are fundamental to understanding polyploid taxa and interspecfic hybrid relationships. And of course, the application of rapidly evolving and increasingly sophisticated molecular investigative tools is required in a systematic basis, not just in regards to individual taxa or species complexes.

Despite the laudable achievements of pioneering 19th and 20th Century investigators and the remarkable advances of recent decades, the potential for the identification of new taxa remains high. Indeed, one could accurately state that such potential exits *because* of those pioneering efforts. Regardless, it is clear that previously undetected *Isoetes* taxa can be expected just about anywhere in the world where these curious - and 'crazy' - plants are to be found.

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SHORT BIOGRAPHIES OF THE AUTHORS

Daniel Brunton was born in Ottawa, Canada on 17 May 1948. He grew up in that area and graduated with a Geography degree from Carleton University, Ottawa, in 1973. A keen naturalist from an early age, he translated that interest into summer employment and then permanent work in provincial parks in Ontario and Alberta (1967-1979), involved in the areas of environmental interpretation, ecological inventories and systems planning.

The lessons learned in parks prepared him for a career as an independent ecological consultant from which he retired (mostly) in 2017. Encouragement from federal government scientists in Ottawa and insights gained from expert associates in those field endeavors encouraged his interest in natural history generally and botany in particular.

Isoetologists (left to right) Carl Taylor, Angelo Troia and Daniel Brunton visiting bedrock outcrop *Isoetes* sites (*I. piedmontana* is abundant in the flooded erosion pits visible behind Taylor) at Clover, South Carolina, USA, 2 April 2016 (Photo: Gerry Taylor).

An extensive series of field based studies across Canada resulted in numerous publications on rare flora and fauna, especially pteridophytes, from the 1970s through the 1990s and the development of a very large (20,000 specimen) private herbarium.

Association with the late Donald M. Britton, foremost Canadian pteridologist, drew Brunton into the challenging - perplexing! - world of 'Isoetology' in the late 1980s. Beginning with their discovery of an undescribed hybrid, the Britton and Brunton team explored *Isoetes* throughout North American and beyond. They generated numerous papers between 1989 and 2006, describing over a dozen new species, hybrids and subspecies. To date, Brunton has produced 49 publications dealing with the taxonomy, diversity, distribution, ecology and conservation of *Isoetes* in North America, Asia and Europe.

Angelo Troia was born in Palermo, Italy on 3 September 1967 and grew up in Sicily. After obtaining a degree in Natural Sciences at the University of Palermo in 1991 and doing his (obligatory) service in the Italian Navy, he undertook PhD studies in Plant Biosystematics and Ecology between Florence and Bologna (Italy). His doctoral thesis was on the systematics and biogeography of the rare insular endemic *Cytisus aeolicus* Guss. (Fabaceae). Returning to Sicily in 1997, he was introduced to the fantastic world of Mediterranean quillworts by the late Prof. Enrico Bellini, his studies focussing on caryological, taxonomic and ecological aspects of various species.

At the IX OPTIMA Meeting in Paris, in May 1998, Troia and Bellini presented a poster about the (previously unknown) chromosome number of the Mediterranean *Isoetes durieui*. After a period as Director of the Trapani Saltworks Nature Reserve in western Sicily, he returned to academic research in 2007, working as a botanical researcher at the University of Palermo. There he prepared the treatments for the three lycophyte families for the project "Flora Critica d'Italia".

Troia's ongoing research is focused on the diversity, ecology and biogeography of plants, with a particular focus on the genus *Isoetes* (and other lycophytes and ferns), freshwater plants and their habitats, the role of islands in the ecology of the Mediterranean, and a wide variety of conservation issues. In the course of his studies, he has produced 27 publications involving the taxonomy, biogeography, cytology, ecology and conservation of *Isoetes*. His most recent area of research is the study of Characeae (green algae).

SHORT NOTE

TWO NEW COMBINATIONS IN GRAMMITID FERNS (POLYPODIACEAE) FROM NEW GUINEA: *PROSAPTIA LEDERMANNII* AND *RADIOGRAMMITIS HABBEMENSIS*

Two new combinations in New Guinea Grammitid ferns (Polypodiaceae) are proposed: *Prosaptia ledermannii* (Brause) Parris and *Radiogrammitis habbemensis* (Copel.) Parris. Herbarium abbreviations follow Thiers (2018).

***Prosaptia ledermannii* (Brause) Parris, comb. nov.**, based on *Davallia ledermannii* Brause, Bot. Jahrb. Syst. 56: 123 (1920). Type: Nordöstl. Neu-Guinea, Kaiserin-Augusta-Fluss-(Sepik-)Gebiet, Schraderberg, 2070 m alt., 31 May 1913, *Ledermann* 11842 (BM! image). Syn. *Polypodium subhamatopilosum* Alderw., Bull. Jard. Bot. Buitenzorg, sér. 3, 5: 220 (1922), *nomen novum pro Davallia ledermannii* Brause, *non Polypodium ledermannii* Brause (1920); *Prosaptia subhamatopilosa* (Alderw.) Ching, Bull. Fan Mem. Inst. Biol. 10: 242 (1941); *Ctenopteris ledermannii* (Brause) Copel., Univ. Calif. Publ. Bot. 18: 225 (1942); *Grammitis subhamatopilosa* (Alderw.) Christenh., Global Flora GLOVAP Nomenclature Part 1, Vol. 4: 55 (2018).

***Radiogrammitis habbemensis* (Copel.) Parris, comb. nov.,** based on *Grammitis habbemensis* Copel., Univ. Calif. Publ. Bot. 18: 223 (1942). Type: Netherlands New Guinea, 9 km east of Lake Habbema, 2860 m, Oct. 1938, *Brass* 10520 (holotype MICH!; isotypes A!, BO!, BRI, K! L! Syn. *Oreogrammitis habbemensis* (Copel.) Parris, Gard. Bull. Singapore 58(2): 260 (2007).

A detailed examination of the second collection of this species (*Kessler* 14049 & *Abrahamczyk*, Indonesia, Papua Barat, road from Wamena to Lake Habbema, 6 km before summit, 4°08'S 138°47'E, 3000 m, 20 March 2011, UC) shows that the rhizome is radial with stipes arranged in whorls, characteristic of *Radiogrammitis* Parris rather than dorsiventral, with stipes in two rows, characteristic of *Oreogrammitis Copel.,* so the species needs to be transferred to the former from the latter.

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Continued on page 347

GROWTH OF FERN GAMETOPHYTES AFTER 20 YEARS OF STORAGE IN LIQUID NITROGEN

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Key words: cryopreservation, *ex situ* conservation, gametophyte; *in vitro;* long-term storage

ABSTRACT

In vitro grown gametophytes of six species of ferns, which had been cryopreserved using the encapsulation dehydration procedure, were evaluated for survival after 20 yrs of storage in liquid nitrogen. Tissues were rewarmed and transferred to a recovery medium with the same methods originally used to test pre-storage viability. All six species resumed growth. Post-storage viability was not consistently higher or lower than pre-storage viability of LN exposed tissues, likely reflecting the small sample sizes. However, these results demonstrate that long-term storage in liquid nitrogen is a viable option for preserving gametophytes of at least some fern species and could be utilized as an additional tool for preserving valuable gametophyte collections and for the *ex situ* conservation of fern biodiversity.

INTRODUCTION

For many species of ferns, gametophyte tissues have proven to be highly adaptable to growth *in vitro* (Table 1). Most of these have been initiated through the aseptic germination of spores, although the aseptic germination of gemmae has also been demonstrated (Raine & Sheffield, 1997). As *in vitro* cultures, gametophytes can provide tissues for research and for propagation, both for ornamental ferns as well as for ferns of conservation concern. The *ex situ* conservation of ferns has traditionally relied on living collections and spore banks (Ballesteros, 2011). However, there are situations where it may be desirable to conserve other types of fern tissues, particularly if spores are few or are difficult to access. If only a few spores are available, they can be germinated *in vitro* and multiplied to provide a theoretically unlimited supply of gametophytic tissue, which can be used to generate sporophytes.

Tissues of such *in vitro*-grown gametophytes have also been shown to be adaptable to storage using cryopreservation, or storage in liquid nitrogen (LN) (Barnicoat *et al.*, 2011; Makowski *et al.*, 2016, 2015, Mikula *et al.*, 2010; Mikuła, Jata & Rybczyski, 2009; Pence, 2015; 2000; Wilkinson, 2002). This can serve as an alternative to maintaining active cultures, which requires a constant input of labor and resources. Gametophytes appear to be particularly adaptable to cryostorage, largely due to their simplicity of form and high regenerability (Maeda & Ito, 1981). However, results thus far have demonstrated the ability of gametophytic tissues to survive only short-term exposure to LN, generally 1 hr-3 days (Mikula *et al.*, 2010; Pence, 2000).

In this lab in the 1990s, tissues from *in vitro* cultures of gametophytes of several fern species were shown to survive LN exposure (Pence, 2000). These common species were chosen because they provided readily accessible material for experimentation, but three

TABLE 1.

Species of ferns that have been grown as gametophytes *in vitro*, some of which have also survived exposure to LN.

Species	Family	Method[a] if LN Exposed	Reference
Adiantum capillus-veneris L.	Adiantaceae		Somer *et al.*, 2009
Adiantum tenerum Sw.	Adiantaceae	ED	Pence, 2000
Adiantum trapeziforme L.	Adiantaceae	ED	Pence, 2000
Anemia phillitidis (L.) Sw.	Schizaeaceae		Kaźmierczak, 2003
Asplenium adiantum-nigrum L.	Aspleniaceae		Somer *et al.*, 2009
Asplenium scolopendrium L.	Aspleniaceae	ED	Pence, 2015
Ceratopteris richardii Brongn.	Parkeriaceae		Kamachi & Noguchi, 2012
Ceratopteris thalictroides (L.) Brongn.	Parkeriacae	ED	Makowski *et al.*, 2015
Ceterach officinarum DC.	Aspleniaceae		Somer *et al.*, 2009
Cibotium glaucum (Sm.) Hook. & Arn.	Dicksoniaceae	ED, EV, V	Mikula *et al.*, 2010; Pence, 2000
Cibotium schiedei Schltdl. & Cham.	Dicksoniaceae	ED, EV, V	Mikula *et al.*, 2010
Ctenitis pauciflora (Kaulf.) Holttum	Dryopteridaceae	ED	Barnicoat *et al.,* 2011
Cyathea australis Domin.	Cyatheaceae	ED, EV, V	Mikula *et al.*, 2010; Mikuła, Jata & Rybczyski, 2009
Cyathea dealbata (G.Forst.) Sw.	Cyatheaceae	ED, EV, V	Mikula *et al.*, 2010
Cyathea delgadii Sternb.	Cyatheaceae	ED, EV, V	Mikula *et al.*, 2010
Cyathea smithii Hook.f.	Cyatheaceae	ED, EV, V	Mikula *et al.*, 2010
Davallia canariensis (L.) Sm.	Davalliaceae		Somer *et al.*, 2009
Davallia fejeensis Hook.	Davalliaceae	ED	Pence, 2000
Dicksonia fibrosa Colenso	Dicksoniaceae	ED, EV, V	Mikula *et al.*, 2010

Dicksonia sellowiana (Pr.) Hook.	Dicksoniaceae		Fiori, Santos & Randi, 2009
Drynaria quercifolia (L.) J.Sm.	Polypodiaceae	ED	Pence, 2000
Dryopteris dilatata (Hoffm.) A.Gray	Dryopteridaceae		Somer *et al.*, 2009
Dryopteris filix-mas (L.) Schott	Dryopteridaceae		Somer *et al.*, 2009
Hymenophyllum tunbrigense (L.) Sm.	Hymenophyllaceae	ED	Wilkinson, 2002
Lepisorus longifolius (Blume) Holttum	Polypodiaceae	ED	Barnicoat *et al.,* 2011
Macroglossum smithii (Rac.) Campbell	Marattiaceae	ED	Barnicoat *et al.*, 2011
Notholaena R.Br. 'Sun Tuff'	Adiantaceae		Rogers & Banister, 1992
Osmunda regalis L.	Osmundaceae	ED, EV, V	Makowski *et al.*, 2016 Mikula *et al.*, 2010
Osmundastrum cinnamomeum (L.) C.Presl	Osmundaceae		Hollingsworth *et al.*, 2012
Phlebodium aureum (L.) J.Sm.	Polypodiaceae	ED	Pence, 2000
Phyllitis (Asplenium) scolopendrium (L.) Newman	Aspleniaceae	ED, EV, V	Mikula *et al.*, 2010
Polypodium cambricum L.	Polypodiaceae		Bertrand *et al.,* 1999; Somer *et al.,* 2009)
Pteridium aquilinum (L.) Kuhn	Dennstaedtiaceae		Whittier & Steeves, 1960
Pteris adscensionis Sw.	Pteridaceae	ED	Barnicoat *et al.,* 2011
Tectaria devexa (Kunze) Copel.	Dryopteridaceae		Ashmore *et al.*, 2011
Thelypteris confluens (Thunb.) C.V.Morton	Thelypteridaceae		Sara & Manickam, 2007
Pronephrium triphyllum (Sw.) Holttum	Thelypteridaceae		Marimuthu & Manickam, 2011
Sphaerostephanos unitus (L.) Holttum	Thelypteridaceae		Marimuthu & Manickam, 2011
Trichomanes speciosum Willd.	Hymenophyllaceae		Raine & Sheffield, 1997
Woodwardia radicans (L.) Sm.	Blechnaceae		DeSoto *et al.*, 2008

[a]Methods: ED = encapsulation dehydration; EV = encapsulation vitrification; V = vitrification

of the species are also congeneric with rare species (Kang et al., 2008; Kishida, 2015; Yu et al., 2017). In those studies, in addition to testing the initial survival of the gametophytes (Pence, 2000), samples were banked in the Frozen Garden™ of CREW's CryoBioBank™ for long-term storage. In a recent study to evaluate the viability of numerous samples from the Frozen Garden after years of storage, several samples of gametophytes of these six species were removed and evaluated for their viability after 20 yrs in LN. The results of that study are presented here.

METHODS

In vitro gametophyte cultures of *Davallia fejeensis* Hook., *Drynaria quercifolia* (L.) J. Sm., *Cibotium glaucum* (Sm.) Hook & Arn., *Adiantum trapeziforme* L., *Adiantum tenerum* Sw., and *Phlebodium aureum* L. were originally initiated by the aseptic germination of spores *in vitro*, as previously described (Pence, 2000), and maintained on a medium of half-strength Murashige and Skoog salts and minimal organics (Linsmaier and Skoog, 1965) with 1.5% sucrose and 0.22% Phytagel (gellan gum, Sigma Chemical Co., St. Louis, MO) (= ½ MS medium), in 60 x 15 mm Petri plates (Corning™ Falcon™, Durham, NC), approximately 15 ml of medium/plate. Cultures were maintained at 26^{o}C under cool white fluorescent lights at 10 – 20 µmol m^{-2} sec^{-1} PPFD, with a 16:8 hr light:dark cycle.

Tissue banking took place between 6/21/1995 and 7/26/1995. Fragments of gametophyte tissue were isolated by cutting the gametophyte with a scalpel into small pieces from throughout the gametophyte thallus. Pieces ranged in size from 1-3 mm in length and width. Gametophyte fragments were prepared for cryopreservation by culturing for seven days on ½ MS medium with the addition of filter-sterilized 10 µM abscisic acid (ABA). They were then cryopreserved using the encapsulation dehydration procedure (Fabre & Dereuddre, 1990), in which the fragments were encapsulated in alginate beads before an18 hr incubation in a 0.75 M sucrose solution, with more than one fragment being encapsulated per bead. The encapsulated tissues were then dried for 3 or 5 hrs under the airflow of a laminar flow hood. The dried beads were stored in 2 ml polypropylene cryovials (Corning®, Corning, NY), 6 to 19 beads per vial, the number of beads varying depending on the amount of tissue available. The samples were stored in a MVE XC 47/11, 900 vial capacity LN storage tank (Chart MVE, Ball Ground, GA), submerged in LN (-196^{o}C). As a control at the time of banking, one vial of each species from each time of banking was rewarmed after a one-day exposure to LN and cultured on ½ MS medium for recovery. Recovery growth was scored as the number of beads showing green growing tissues, and data for the samples were taken between nine and 28 days after rewarming. In the case of three species, survival was also measured after 4.5 months of growth, with data taken after four weeks of growth. Samples from each species were also removed after 3.5 yrs of storage in LN, and recovery data was taken after two and seven weeks of growth. The data taken seven weeks after rewarming were reported previously (Pence, 2000).

Samples were removed from LN between 7/16/2015 and 8/14/2015 to evaluate 20-yr survival. Recovery conditions in the present study replicated as much as possible the original recovery conditions, using ½ MS medium, only with modification in the source of the prepared MS salt mixture (Phytotechnology Laboratories, Overland Park, KS) and the source and amount of the gellan gum (Gelzan, Caisson Labs, Smithfield, UT), added at 0.33%. One vial representing each species and each banking time for that species was removed (9 vials total) and rewarmed at ambient temperature (22^{o}C) for 15 min. The

beads were then plated on 1 – 4 plates of ½ MS medium, depending on the number of beads, 4-6 beads/plate. Cultures were maintained under the same temperature and lighting conditions originally used for growth.

At the time of banking and at 3.5 yrs, survival was scored as the number of beads showing some recovery growth of encapsulated tissues, regardless of the number of fragments per bead. When tissues were evaluated after 20 years, survival was scored both as the percent of fragments and the percent of beads showing green, growing tissue at 3 – 4 weeks.

RESULTS

Growth from cryopreserved gametophyte tissue occurred from all of the species of ferns tested in this study (Table 2). The values for the original survival data reported here are those recorded for the specific samples that were banked and are different from those reported previously (Pence, 2000), which were collected from experiments done in preparation for the long-term banking of samples. In all but two species, the percent of tissues showing growth after storage was greater than that observed before long-term storage. In *Drynaria quercifolia* and both samples of *A. trapeziforme,* post-storage growth was less than that observed originally, and in one sample of *A. trapeziforme,* there was no post-storage growth. However, that sample produced fungal growth on a few beads within the first few days of culture, and the remaining beads were transferred to fresh plates. The transferred beads did not show any contamination, but they also did not show any growth. Specific moisture levels of the banked samples used in this study are not available, except for two of the three samples, *D. quercifolia* and *A. trapeziforme,* which were dried for 5 hrs to 17.5% and 18.9% moisture, respectively.

Samples recovered at 20 yrs were scored at 3 – 4 weeks. When these were compared with samples recovered at 3.5 yrs, the percent survivals were, in most cases, more similar to those recorded at two weeks from the 3.5 yr samples than with survivals recorded at seven weeks for those samples, which in all cases were higher than two week survival values (Table 2).

Since pre-storage viability was based on growth per bead, post-storage measurements were calculated both on growth per bead and on growth per fragment for seven of the nine samples. The percent survival was very similar between the two measurements (Table 2). Most fragments showed some damaged areas, but ultimately, those with any surviving areas went on to regenerate gametophyte tissues (Figure 1).

DISCUSSION

These results demonstrate that *in vitro*-grown gametophytes of at least some fern species can survive up to 20 yrs in LN storage without appreciable deterioration using the encapsulation dehydration procedure. This method has been applied by others in the intervening yrs with good success (Table 1). There also have been reports of some survival of fern gametophytes using the encapsulation vitrification method (Makowski *et al.*, 2016; Mikula *et al.*, 2010), although when it was compared with encapsulation dehydration, the latter resulted in higher survival. Studies using vitrification without the protection of encapsulation have uniformly reported severe plasmolysis of the gametophyte tissues and no survival after exposure to LN (Makowski *et al.*, 2016; Mikula *et al.*, 2010).

When the successful cryopreservation of fern gametophytes was reported originally, 3 – 4 hrs of drying brought samples to 19-27% moisture (wet weight basis) (Pence, 2000).

TABLE 2

Percent survival of six species of fern gametophytes, dried for 3 or 5 hrs, through LN exposure before storage and after 4.5 mos, 3.5 yrs, and 20 yrs of LN storage. Day of observation is given in adjacent column, if different for different samples, or in the heading, if the same for all samples.

Species	Hrs of Drying	Pre-storage		4.5 Mos	3.5 Yrs		20 Yrs		
		Day	Survival	[a]Survival (at 4 wks)	Survival (at 2 wks)	[b]Survival (at 7 wks)	Day	Survival per Bead	Survival per Piece
Adiantum tenerum	3	12	**41.7**		**63.0**	**88.0**	28	**66.7**	**66.7**
	3	16	**60.0**	**73.3**			28	**80.0**	**79.5**
Adiantum trapeziforme	3	12	**25.0**		**33.0**	**50.0**	28	**0.0**	**0.0**
	5	4	**84.6**				20	**39.3**	**nd**
Cibotium glaucum	3	16	**46.2**	**80**	**60.0**	**75.0**	28	**90.0**	**91.7**
Davallia fejeensis	5	8	**46.2**		**80.0**	**100.0**	28	**82.3**	**82.3**
	3	9	**6.7**	**40**			20	**50.0**	**nd**
Drynaria quercifolia	5	6	**91.7**		**50.0**	**67.0**	28	**41.3**	**40.5**
Phlebodium aureum	3	27	**19.3**		**67.0**	**90.0**	28	**83.5**	**83.5**

[a]Data available for three species only.
[b]Seven week survival data from Pence, 2000.

The moisture contents available for two of the samples in this study that had been dried for five hrs were 18 – 19%. With seeds, a number of studies have been directed at defining optimum storage moisture levels, which are related to the seed oil content (e.g. Pritchard, 1995; Hor *et al.*, 2005). Whether the moisture levels used is this study are optimum for fern gametophytes will require further research, but they were sufficient to allow regeneration of at least some of the tissues after cryostorage.

The fact that survival after long-term storage was greater in most cases than pre-storage survival may have resulted from data being taken much earlier in the case of some of the pre-storage samples, as these were done by different operators, at different times, for various goals. The results of the 3.5 yr samples indicate that the initiation of growth continued for several weeks, with more fragments growing at seven weeks than at two weeks. Alternatively, the sample sizes in this study were small and may not be large enough to clearly track any small changes in viability over the time of storage. Similar increases in viability after LN exposure have been noted with small samples of other species, by others, as well as in this laboratory (Mix-Wagner, Schumacher & Cross, 2003; Vanhove, Philpott & Pence, 2016).

The highly regenerative nature of fern gametophytes has been noted for several species (Maeda & Ito, 1981; Ong & Ng, 1998), and this ability makes these tissues well adapted to cryopreservation. Even if some tissues are damaged in the process of either cryoprotection or LN exposure, only a small amount of tissue is required (possibly only one cell) to regenerate the gametophyte tissue (Maeda & Ito, 1981). However, this ability makes it more difficult to evaluate survival quantitatively, because fragments are of different sizes and only portions of them may survive. Having more than one fragment encapsulated in an alginate bead can increase the number of fragments available for regrowth and minimize the number of beads needed, and survival per bead appears to

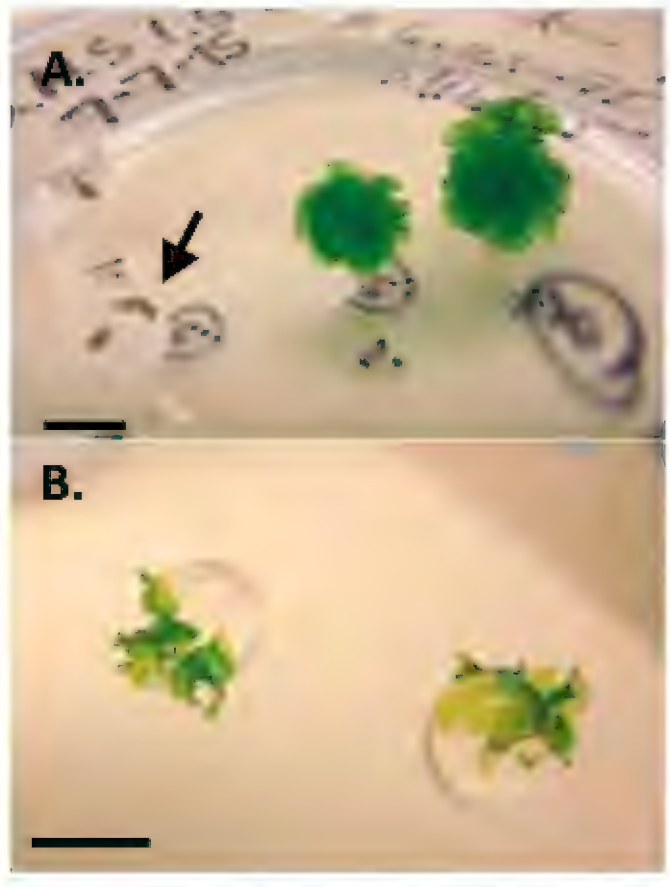

Figure 1. Encapsulated gametophtyes from two fern species resuming growth after 20 yrs of storage in liquid nitrogen. A. *Adiantum tenerum* gametophytes, as well as fragments that did not grow (arrow); B. *Phlebodium aureum* gametophytes. Scale bars = 0.5 cm.

correlate well with fragment survival. When survival of the 20-yr old samples was measured by both methods, the results were very similar.

Cryopreservation of fern gametophytes has thus far been applied to *in vitro* cultures of gametophytes. This requires the initiation of gametophyte cultures by the aseptic germination of spores or gemmae, and *in vitro* fern gametophyte cultures initiated in this way have been established and reported for a number of species (Table 1). However, for species known only from the gametophyte in nature, *in vitro* cultures would need to be established directly from gametophytic tissue. Methods for this have been reported for gametophytes of bryophytes (Rowntree & Ramsay, 2005; Segreto *et al.*, 2010) but not yet for fern gametophytes. Gametophytes of bryophytes are more complex structures than those of ferns, and further research is needed to determine whether fern gametophytes can survive the surface sterilization required for initiating *in vitro* cultures.

The results of this study indicate gametophyte cryopreservation is an option for long-term conservation of *in vitro* collections of fern gametophytes that have been developed for horticultural or experimental purposes, as well as an option for the *ex situ* conservation of fern biodiversity. Thus, it is a tool that can contribute to the *ex situ* conservation goals (Target 8) of the Global Strategy for Plant Conservation (Convention on Biological Diversity, 2010). Further research is needed to test the applicability of encapsulation dehydration to more species with different habits and adaptations in order to more clearly define the breadth of its potential use. In addition, more long-term studies should be initiated to evaluate the effectiveness of cryopreservation in preserving tissues over the course of decades. However, these results with six species clearly demonstrate the potential of this method for fern conservation in the future.

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